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(54) Title: DNA PROBES AND PRIMERS FOR DETECTION OF B. BURGDORFERI USING THE POLYMERASE CHAIN REACTION

(57) Abstract

Nucleic acid primers of complementary sequence to portions of the gene encoding a semiconserved region of *B. burgdorferi* flagellin are utilized to amplify target sequences of nucleic acids extracted from infective sites of Lyme borreliosis, which are then probed with a nucleic acid tracer conjugated to signal-generating molecules for highly specific detection of *B. burgdorferi*.

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DNA PROBES AND PRIMERS FOR DETECTION OF B. BURGDORFERI
USING THE POLYMERASE CHAIN REACTION

This application is a continuation-in-part of my prior copending application serial number 7/538,957, filed June 15, 1990.

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FIELD OF THE INVENTION

The present invention relates to nucleic acid probe assays for the detection of infectious agents present in biological samples in minute amounts.

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BACKGROUND OF THE INVENTION

Lyme disease is a complex of clinical disorders caused by the tick-borne spirochete *Borrelia burgdorferi*. It is the most common arthropod-borne disease in North America and Europe. Distinctive forms of the infection include erythema chronicum migrans, acrodermatitis chronica atrophicans, lymphocytic meningoradiculitis, and Lyme arthritis. Serious nervous system or cardiac involvement is observed in the second and third stages of the disease.

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Variations in clinical presentation and the disease's well-known mimicry of other neurological and rheumatological disorders make definitive diagnosis difficult. For a general review of the clinical and etiological aspects of Lyme disease, see Steere, et al., N. Engl. J. Med. 308:733 and Duray, Rev. Infect. Dis. 112:S1487. In spite of the extremely serious clinical course of Lyme disease, it responds very well to antibiotic treatment, if diagnosis and proper treatment can be instituted early. Early diagnosis, however, is frustrated by the unavailability of tests capable of reliable differential diagnosis within the

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first days or weeks post-infection. The diagnostic tests in current use are all serologic, and include indirect immunofluorescence (IFA), enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Such immunologic tests are unreliable because only about 50-60 percent of patients with early disease have diagnostic titers measurable by either IFA or ELISA during the first three to six weeks post-infection.

In addition, false positive reactions in IFA and ELISA have been reported in patients having syphilis caused by *T. pallidum*, and relapsing fever caused by *Borrelia hermsii*. The prevalence of false positives in serologic based assays for *B. burgdorferi* is attributable to the high degree of genetic interrelationship amongst pathogenic spirochetes resulting in serological cross-reactivity of the major structural determinants. See, for example, Magnarelli, et al., J. Infect. Dis., 156:183 (1987).

An alternative to serologic assays is a test based upon the property of nucleic acid probes to bind very specific target sequences. Since in Lyme disease the organism itself is present during infection in very low numbers, it is necessary to first amplify the target nucleic acid sequences so that the probes to which a signal generating molecule is attached, hybridize in sufficient number to generate a detectable signal. The strategy of such probe based assays is that the target sequence be specific for the species of organism sought to be detected, and recognition of

target sequences encompass every member strain of the species.

A nucleic acid probe assay for *B. burgdorferi* must be able to distinguish between this organism and closely related species, in particular variants of relapsing fever, *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. hispanica*, and *B. recurrentis*. DNA homology studies on a variety of *Borrelia* indicate varying degrees of homology, with *B. hermsii* showing the greatest homology at 58 to 63 percent, as reported in Hyde and Johnson, Zbl. Bakt. Hyg. A, 263:119 (1988). Three North American species, *B. hermsii*, *B. turicatae* and *B. parkeri* share 77 to 100 percent homology, but comparison of European and North American strains indicates substantial intra-species variation.

In the detection of *B. burgdorferi* by nucleic acid probe assays, attention has focused on probes for the OspA and OspB surface protein antigens. Nielsen, et al., Mol. Cel. Probes, 4:73 (1990) reported successful probing of an amplified 145 base pair segment of OspA to detect as little as 50 fg of *B. burgdorferi* DNA corresponding to 50 spirochetes. The probe was found to be specific for *B. burgdorferi* in that no cross-reactivity was observed for *B. hermsii*, *T. pallidum*, *T. denticola*, or *S. aureus*. However, Persing, et al., J. Clin. Micro., 28:566 (1990) probed the OspA region of a number of bona fide *B. Burgdorferi* isolates, and found that the probes were unable to positively identify all of the isolates. The molecular basis of this result was the finding that some *B. burgdorferi* strains possess an

extensively different OspA protein or lack the OspA protein altogether, and possess instead a smaller protein termed pC. Hence, at present there is no definitive assay for *B. burgdorferi*.

More recent studies indicate that *B. burgdorferi* may comprise more than one group of organisms. Postic et al., Res. Microbiol., 141: 465 (1990) analyzed thirteen strains from diverse sources by DNA/DNA hybridization, and found that a subgroup of four strains could be distinguished from nine reference strains. Rosa, et al. J. Clin. Microbiol., 29: 524 (1991) also found that two subgroups of *B. burgdorferi* could be identified by DNA amplification patterns using a *B. burgdorferi* specific target sequence from an unknown portion of the genome. It should be noted that there were major discrepancies between the results of these two groups, indicating that these systems may not have satisfactorily specific diagnostic value.

20 SUMMARY OF THE INVENTION

Comparison of the DNA sequences encoding the highly conserved flagellin genes from *B. burgdorferi* and *B. hermsii* reveal a small region of relative non-homology containing 50 to 70 percent mismatched bases including short deletions. Primers selected from this region were found to direct *B. burgdorferi* specific amplification of the interprimer sequence. Probes constructed for the interprimer region specifically identify *B. burgdorferi*.

In accordance with the present invention, a bioassay utilizing such primers and probes for

specific detection of *B. burgdorferi* in a biological specimen comprises

extracting the nucleic acid fraction from the specimen,

5 adding primers of complementary strand specificity to sequences flanking a semiconserved region of nucleic acid encoding the flagellar open reading frame

10 hybridizing the primers to the complementary strand sequences

amplifying the semiconserved region by nucleic acid amplification techniques

15 hybridizing the amplified semiconserved region to a signal-generating probe constructed from the interprimer sequence, and

detecting the signal emitted by the hybridized probe.

In another aspect of the present invention, oligonucleotide primer pairs are selected for 20 specificity to *B. burgdorferi* in the flagellar open reading frame region, a first such primer comprising a sequence of about 8-32 consecutive bases from nucleotide numbers 390 to 712 of the flagellar open reading frame, and a second such 25 oligonucleotide primer comprising a sequence on the complementary strand located spacedly at least 50 nucleotides from the 5' end of the first primer comprising 8-32 substantially consecutive bases having not greater than 70 percent homology with the corresponding sequence of *B. hermsii*.

30 A further aspect of the invention involves the selection of probes for specific detection of nucleic acids amplified from the non-homologous

region of the flagellin gene open reading frame. These probes comprise signal-generating oligonucleotide tracers substantially homologous to a sequence of the flagellar open reading frame of *B. burgdorferi*, but only about 70 percent homologous to the corresponding sequence of *B. hermsii*. Signal-generating means conjugated to the tracers may be radiolable, a fluorescent label, or a biotinylated label.

A probe containing an oligonucleotide having the sequence CTC TGG TGA GGG AGC TCA AAC TGC TCA GGC TGC ACC GGT TCA AGA GGG T, was used to screen a number of *B. burgdorferi* strains from which amplifications of the interprimer sequence had been carried out. The probe detected some amplified nucleic acids, but not others. Accordingly, it is a further aspect of the present invention to utilize the sequences of these interprimer regions as targets for detection of subgroups of *B. burgdorferi*. One variant sequence of the *B. burgdorferi* flagellin gene open reading frame hypervariable region extending from nucleotide number 517 to number 742 comprises base substitutions of a guanosine at positions 531, 549, 552, 612, 613, 684, 693, adenosine at positions 539, 591, 603, 622, 639, 651, 666, thymidine at positions 540, 573, 630, 696, and cytosine at position 735. Another variant sequence of the *B. burgdorferi* flagellin gene open reading frame hypervariable region extending from nucleotide number 517 to number 724 comprises base substitutions of a guanosine at positions 531, 595, 612, 649, and 663, adenosine at positions

539, 552, 651, 666, 670, and 688, thymidine at positions 540, 571, 594, 630, and 696, cytosine at positions 644 and 726. Finally, a set of probes complementary to a portion of the target sequences comprise an oligonucleotide tracer for detection
5 of subgroups of *B. burgdorferi* comprising an oligonucleotide probe having a sequence selected from the group consisting of CTC TGG TGA GGG AGC TCA AAC TGC TCA GGC TGC ACC GGT TCA AGA GGG T, CTC TGG TGA AGG AGC TCA GGC TGC TCA GAC TGC ACC TGT TCA AGA AGG, and TGC TGG TGA GGG AGC TCA AGC TGC TCA GGC TGC ACC TGT TCA AGA GGG TGC, and signal means conjugated to said probe.

10 **DETAILED DISCLOSURE OF THE PREFERRED EMBODIMENT**

15 In prior attempts to develop a probe based assay for amplified target sequences of *B. burgdorferi*, the major surface antigen proteins expressed from the OspA and OspB genes were selected. Since not all bona fide *B. burgdorferi* strains are found to encode these antigens, it is
20 apparent that a different sequence must be selected.

25 In spirochetes, the endoflagellum is composed of an assemblage comprising a single subunit. Wilske, et al., Zbl. Bakt. Hyg. A 263:92 (1986) reported that monoclonal antibodies isolated against the flagellin protein of *B. burgdorferi* also reacted with *B. hermsii*, *B. duttonii*, *B. turicatae* and *B. parkeri*. Because of its unitary
30 subunit structure, evidence of antigenic homology, and the combination of both structural and physiological constraints within the same molecule, the flagellin gene may be highly

conserved, and therefore be likely, as a target in
a probe based assay, to identify every member of
the species. Cross-reaction of several species to
the same antisera, however, also suggest that
these genes may be highly conserved between
species in the same genus.

5 Isolation and sequencing of the flagellin
gene for *B. hermsii*, the most closely related
species to *B. burgdorferi*, in fact, revealed large
regions of homology with *B. burgdorferi*.

10 Unexpectedly, however, there was also a region of
approximately 480 base pairs within the open
reading frame of the flagellin gene which contains
sequences of relative non-homology. In the
15 present invention, these sequences of relative
non-homology provide the basis for constructing
primers and probes which are specific in
amplifying and detecting *B. burgdorferi*, but which
distinguish other closely related species.

20 Figure 1 shows the homology comparison of the
nucleotide sequences of the flagellin genes from
B. burgdorferi and *B. hermsii*. Alignment of the
nucleotides was assisted by the UWGCGT analysis
program BESTFIT, as referenced in Nuc. Acids Res.,
25 12:126 (1988). The *B. burgdorferi* sequence is
positioned above the *B. hermsii* sequence. It is
apparent from the comparison that the region of
non-homology is bracketed by nucleotides at
approximately positions 390 and 712. The region
30 from about nucleotide position 475 to 770 are
preferred for primer and probe construction
because it contains at least two small deletions
in the *B. hermsii* sequence.

In selecting primer sequences, it is desirable to utilize oligonucleotides for preferably about 8 to 32 nucleotides. Use of less than 8 nucleotides reduces specificity and decreases binding affinity. Use of greater than about 32 nucleotides does not enhance the priming function or increase specificity, and may increase the probability of binding in regions of significant mismatch. However, there will be sequences within the non-homologous region in which a primer of 6 or 7 nucleotides, or greater than 32 would be operable, and are to be considered the equivalents of the preferred sequence length.

The strategy in primer selection is to identify short sequences native to *B. burgdorferi* which are maximally non-homologous to the corresponding sequences of *B. hermsii*. In the amplification reaction, the primers will anneal only to the *B. burgdorferi* template and not to *B. hermsii* DNA, so that no specific amplification of sequences hybridizable to the signal-generating probe will occur. Additionally, primers are selected so as to flank the target region on opposite strands. The target region should be at least as long as the probe to which it hybridizes, preferably about 50 nucleotides in length. Thus primer pairs are selected which comprise a first oligonucleotide selected from the group consisting of a sequence comprising about 8-32 substantially consecutive bases from nucleotide numbers 390-712 of the flagellar gene open reading frame, and a second oligonucleotide selected from the group

consisting of a sequence on the complementary strand located spacedly about 50 nucleotides from the 5' end of the first primer, comprising 8-32 substantially consecutive bases having not greater than 70 percent homology with the corresponding sequence of *B. hermsii*.

The selected primers are utilized as initiation points on the denatured nucleic acid template for chain extension on the complementary strand. Conveniently, the amplification of nucleic acids is carried out using the polymerase chain reaction (PCR), as described in U.S. Patent No.s 4,683,195 and 4,683,202 (Mullis). In this method, target DNA is denatured, and primers are hybridized to complementary sequences flanking the target region to be amplified on the respective opposite strands. DNA polymerase is added together with nucleotide triphosphates and new DNA is synthesized from the primer. When the reaction is complete, the DNA is once again denatured, more polymerase is added, and another round of DNA synthesis occurs. By repeating this a number of times, a high degree of amplification on the order of 5-6 logs can be achieved. It is desirable to conduct the hybridizations and polymerizations under as stringent a set for conditions as possible, that is, at elevated temperatures wherein the binding of primers requires even greater fidelity to the exact sequence. The preferred temperature is 65 degrees Centigrade, which requires use of a thermostable polymerase, such as that described in U.S. Patent No. 4,889,818 (Gelfand et al.).

Other protocols for nucleic acid amplification known in the art may be substituted for PCR. One such method, called 3SR, utilizes reverse transcriptase to create an RNA/DNA hybrid from which a duplex DNA structure containing a T7 or other suitable transcriptional promoter is produced. A DNA dependent RNA polymerase is added in the system and a large number of target sequence specific transcripts are synthesized.

These amplified transcripts are then probed with a suitable signal generating oligonucleotide to detect the specific target. In constructing the primers for 3SR or a related amplification method called TAS, the promoter sequence must be conjugated to the 5' end of the primer. The T7 promoter having the sequence 5'-
TAATACGACTCACTATAGGGGA is preferred.

The probe sequence may be selected from any interprimer sequence in the non-homologous open reading frame region. The best probes are those incorporating a sequence of maximum mismatch. In this region, the sequence from about base number 585 to about base number 650 is especially efficacious because it spans the region containing the deletions and has an overall approximately 50 percent mismatch. In the preferred embodiment, selection of a probe rather than a primer or primers from this region is based on the observation that a highly mismatched probe will not hybridize significantly with target at low levels of amplification, thus resulting in a substantially qualitative assay.

The probe length may be varied over a potentially wide range from a few to hundreds of base pairs. However, a balance must be struck between the specificity of the probe and its stability. The shorter probes, particularly those selected from highly non-homologous regions, are more highly specific than longer ones, but tend to be unstable at temperatures that give good hybridizing stringency. Longer probes are less specific generally because they are able to tolerate a greater number of mismatches. The preferred probes are those selected from regions of relative non-homology, have relatively high GC content, and are about 35-55 nucleotides.

Utilizing an oligonucleotide selected from a region encompassed by nucleotide positions 594 to 642 of the flagellin gene open reading frame having the highest degree of mismatch and non-homology, namely, CTC TGG TGA GGG AGC TCA AAC TGC TCA GGC TGC ACC GGT TCA AGA GGG T, a ³²P conjugated probe was constructed. A number of putative *B. burgdorferi* strains were screened by first amplifying the target region extending between the loci within the flagellin gene to which the above-described primers anneal (the interprimer amplification region).

The amplified nucleic acids were then probed with the above probe in a Southern blot assay. Surprisingly, some of the bona fide *B. burgdorferi* strains showed hybridization to the probe, and others did not. The amplified regions were sequenced. The sequences are shown in Figure 6.

These sequences contain several positions of mismatched bases. By selecting a region containing minimally 5 mismatched bases, three probes were obtained which could distinguish three subgroups of *B. burgdorferi* in an assay in which the interprimer region was first amplified, and then the amplified probes were probed with the probes of specific sequence. The three probe sequences are as follows: Probe 1: CTC TGG TGA GGG AGC TCA AAC TGC TCA GGC TGC ACC GGT TCA AGA GGG T, Probe 2: CTC TGG TGA AGG AGC TCA GGC TGC TCA GAC TGC ACC TGT TCA AGA AGG, and Probe 3: TGC TGG TGA GGG AGC TCA AGC TGC TCA GGC TGC ACC TGT TCA AGA GGG TGC.

Table 1 gives the individual subgroups of *B. burgdorferi* strains which are specifically detected by each of the above corresponding probes. Table 2 give the sequences of the 3 subgroup probes.

Applicants have found that the above specific sequences are most preferred because of the stringency of the assay obtained where the extent of mismatched bases is maximized for substantially corresponding regions of the flagellin gene.

However, it will be apparent that minor inconsequential base substitutions may be made which will not adversely affect the specificity of the assay. Such minor sequence variations are considered by Applicants to be the equivalents of the sequences disclosed herein.

The probes in molecular combination with signal generating means become oligonucleotide tracers capable of tagging the target sequence to

Table 1

BORRELIA SPECIES AND STRAINS TESTED

SPECIES <u>B31 SUBGROUP</u>	STRAIN <u>DESIGNATION</u>	GEOGRAPHIC <u>LOCATION</u>	<u>PRIMERS</u>	<u>PROBE 1</u>	<u>PROBE 2</u>	<u>PROBE 3</u>
<i>B. burgdorferi</i>	B31 [ATCC 35210]	New York	+	+	-	-
	IRS [ATCC 35211]	Switzerland	+	+	-	-
	Virulent MM1	Minnesota	+	+	-	-
	Virulent MMT1	Minnesota	+	+	-	-
	Virulent I.pacificus	California	+	+	-	-
	N40	New York	+	+	-	-
	Son 328	California	+	+	-	-
	DN-127	California	+	+	-	-
	Minnesota Mouse	Minnesota	+	+	-	-
	Lake 339	California	+	+	-	-
	France 20001	France	+	+	-	-
	CD16	Minnesota	+	+	-	-
	VS215	Switzerland	+	+	-	-
	NE56	Switzerland	+	+	-	-
	25015	New York	+	+	-	-
	P/Bi	Germany	+	-	-	+
	P/Sto	Germany	+	-	+	-
	P/Gu	Germany	+	-	+	-
	GTI	Germany	+	-	-	+
	G2	Germany	+	-	-	+
	VS3	Switzerland	+	-	-	+
	VS185	Switzerland	+	-	-	+
<i>B. hermsii</i>	HS1 [ATCC 35209]					
	Frogner					
	YOR-1	California	-	-	-	-
	CON-1	California	-	-	-	-
	MAN-1	California	-	-	-	-
<i>B. parkeri</i>			-	-	-	-
<i>B. turicatae</i>			-	-	-	-
<i>B. crocidurae</i>			-	-	-	-
<i>B. anserina</i>			-	-	-	-
<i>B. coriaceae</i>			-	-	-	-
<i>B. coriaceae</i>	Co57 [ATCC 43381]		-	-	-	-

15

Table 2**Probe Oligonucleotides for Specific Detection
of 3 Groups of *Borrelia burgdorferi*****Probe 1 - *B. burgdorferi B31 group***

CTCTGGTGAG GGAGCTAAA CTGCTCAGGC TGCACCGGTT CAAGAGGGT

Probe 2 - *B. burgdorferi P/Sto group*

TGCTGGTGAG GGAGCTCAAG CTGCTCAGGC TGCACCTGTT CAAGAGGGTG CT

Probe 3 - *B. burgdorferi P/Bi group*

CTCTGGTGAA GGAGCTCAGG CTGCTCAGAC TGCACCTGTT CAAGAAGG

which they bind, or of emitting a signal either directly or indirectly. Such tracers may conveniently be a probe conjugated to a radioactive molecule or enzyme. Upon hybridizing 5 of tracer to target, the double-stranded duplex may be separated from unhybridized tracer by conventional separation techniques, and the amount of bound radioactivity measured in a scintillation or gamma counter. Similar conventional separation 10 techniques may be used to separate enzyme-linked hybridized tracers. Upon separation of the enzyme-conjugated duplex, a colorimetric or fluorometric substrate is added, and the signal detected by conventional instrumentation. Other 15 signal-generating systems known in the art include chemiluminescence utilizing acridinium esters or dioxetanes, as described in McCapra, et al., Chemiluminescence and Bioluminescence, Plenum Press, N.Y. (1973), fluorescent tags such as 20 fluorescence assays, or biotin-conjugated probes.

Of particular interest, is the application of fluorescence polarization (FP) which measures the increase in polarization resulting from 25 hybridization of a fluorescein-tagged probe to its target. The importance of this detection system is that detection can proceed immediately upon annealing of tracer to target without first separating unbound tracer. This detection method is described in greater detail in Example 2 hereinbelow.

Utilizing the primers and probes of the present invention, bioassays are conveniently configured for the diagnostic detection of B.

burgdorferi from biological specimens. In such bioassays, the best source of primers and probes is by conventional methods of oligonucleotide synthesis, although purified cloned gene fragments of proper sequence may also be employed.

5 Biological specimens of interest include skin punch biopsies, blood and blood fractions, cerebrospinal fluid, the tick carrier and its body parts, synovial fluid, and fractions of body tissue implicated in the Lyme disease complex.

10 Techniques for punch biopsy specimens are described in Berger, et al., J. Am. Acad. Dermatol., 13:444 (1986). Isolation of spirochetes from blood is described in Benach, et al., N. Eng. J. Med., 308:740 (1983). Isolation of spirochetes from cerebrospinal fluid is described in Karlsson, et al., J. Clin. Microbiol., 28:473 (1990). Isolations from other tissues are also described in the literature.

20 In order to carrying out the amplification step preliminary to the tracer hybridization, it is necessary to extract the nucleic acids to render them available. Extraction methods for obtaining nucleic acids from biological specimens are well-known in the art, and are deemed conventional in carrying out the inventive bioassays. For example, Keller & Manak, DNA Probes, Chapter 2, "Sample Preparation", offer complete details for a number of the standard methods including the original literature references. Further preparation of the nucleic acids by denaturation is also conventional, and

may be effected either by manipulation of pH or salt concentration, or by heating.

The primers, which upon hybridization to the template sequences, act as substrates for polymerase-mediated chain extension, may be added to the reaction mix either before or after denaturation, and annealed upon shifting to renaturing conditions. The polymerase is then added, and successive cycles of chain extension allowed to occur. In PCR, DNATaq polymerase is preferred. In 3SR, reverse transcriptase first creates an RNA/DNA hybrid which upon RNase H digestion and repolymerization with DNATaq polymerase can be repetitively transcribed with DNA dependent RNA polymerase. Methods of detecting the probed amplified nucleic acids have already been described hereinabove.

A particularly powerful method of detecting the hybridized radioactive tracer subjects the products of nucleic acid amplification to agarose electrophoresis, with transfer of the nucleic acid bands to a nylon membrane such as pall biodyne B, followed by hybridization to the signal-generating probe, in a standard Southern blot assay [Southern, J. Mol. Biol., 98:503 (1975)]. The areas of specific hybridization are then visualized by autoradiography of the probed nucleic acid.

Thus, in the bioassays of the present invention, a biological specimen containing suspected sequences of *B. burgdorferi* nucleic acids is nucleic acid extracted and denatured in the presence of primers of complementary strand

specificity to sequences flanking a semiconserved
region of nucleic acid encoding the flagellar open
reading frame, hybridizing the primers to the
complementary strand sequences, amplifying the
5 semiconserved regions by nucleic acid
amplification techniques, hybridizing the
amplified semiconserved region to an
oligonucleotide tracer having a sequence
substantially homologous to a sequence of the
10 flagellar gene open reading frame of B.
burgdorferi but maximally 70 percent homologous to
the corresponding sequence of B. hermsii, and
detecting a signal emitted by signal generating
means of the tracer

15 Further advantages of the present invention
will be apparent from the Example which follows:

EXAMPLE 1

B. hermsii and B. burgdorferi were obtained
from the American Type Culture Collection under
20 accession numbers ATCC 35209 and ATCC 35210
respectively. The cells were grown up and
harvested, and the DNA extracted by conventional
methods. The chromosomal DNA was partially
digested with Sau 3A restriction enzyme, and the
25 fragments characterized on agarose gel
electrophoresis. Suitable sized fragments of 9-23
bp were partially filled in the presence of dGTP
and dATP. This material was then readily cloned
into lambda GEM11 vector, which had been digested
with Xho I restriction nuclease and partially
30 filled in with dTTP and dCTP.

Lambda libraries were then plated using E.
coli LE392 as host. Plates were plaque-lifted

using Pall Biodyne B membranes, and screened by hybridization as in Souther blot analysis.

Synthetic primers prepared at random from the known sequence of *B. burgdorferi*, as reported by Gassman, et al., Nuc. Acid Res., 17:3590 (1989), were utilized to amplify templates derived from *B. burgdorferi* and *B. hermsii*. In one instance a primer pair utilized to generate a 300bp fragment from *B. burgdorferi* quite unexpectedly resulted in a faint band of 300 bp when used to amplify *B. hermsii*. The faint bank identified a slight amplification of a region within the flagellin gene which was otherwise relatively non-homologous to *B. burgdorferi*. Utilizing this fragment as a probe permitted identification of lambda clones containing this portion of the gene. Construction of restriction maps from these clones and sequencing of the constructs, gave the correct sequence for the non-homologous sequence shown in Figure 1.

In a typical experiment, utilizing the primers indicated in Figure 1, amplification of the interprimer region was performed by thirty cycles of PCR. The amplification products were then analyzed as follows: electrophoresed in agarose gel followed by Southern blot analysis. Referring to Figure 2, the left panel shows an agarose gel electrophoresis pattern. The two bands visible together at lower center correspond to the amplification products of two independent strains of *B. burgdorferi*, including a strain negative in amplification studies utilizing amplification of the OspA gene. It is apparent

that the molecular size in each case corresponds to the expected size for amplification of the correct targeted region. The controls are all negative. To confirm that the small bands visible at other segments of the gel for the controls represented nonspecific amplification, the gel was subjected to Southern blot analysis, as shown in the right panel of Figure 2. The transferred nucleic acids were probed with a tracer containing the sequence shown in Figure 1. The results confirm the specificity of the assay.

In a second experiment, the amplified *B. burgdorferi* and control DNA were analyzed in homogeneous assay by hybridization with a probe, fluorescein conjugated at its 5' end having a sequence: 5'GAGCTCCCTCACCAAGAGAAA, by fluorescence polarization. The probe mix contained 1.4 ml of SSPE buffer [Sambrook, et al., Molecular Cloning: a laboratory Manual, 2 Ed., Cold Spring Harbor Laboratory, (1989)], 7 ul of 3.2 M Tris-HCl, 250 fmol probe conjugate, and 200 ul formamide. In a separate tube, a sample of the PCR amplified material was denatured in 0.1 M NaOH for 15 minutes at 55 degrees Centigrade. The sample was then added to the probe mix and fluorescence polarization readings were taken over a 72 minute time period with a Fluorescence Polarimeter.

The results are graphed in Figure 3, and show that specific polarization is qualitatively detected for *B. burgdorferi* samples (designated ATCC#35210 and ATCC#35211 respectively). The controls are negative (ATCC#35209, *B. hermsii*; ATCC#43381, *B. coriaceae*). Referring to Figure 4,

which shows a barograph of the difference in MP (millipolarization units) between the value at 72 minutes and at substantially time zero; it is clear that FP is capable of specifically identifying an amplified target sequence in a homogenous format.

EXAMPLE 2

DNA from the series of *B. burgdorferi* strains listed in Table 1 was extracted. Amplification by polymerase chain reaction (PCR) was carried out as described above, utilizing the primers set forth in Example 1. Subgroup 1 of *B. burgdorferi*, as shown in Table 1 is designated B31. Subgroup 2 is designated P/Bi, and subgroup 3 is designated P/Sto. Probes 1-3, having the sequences shown above, are specific for the Subgroups B31, P/Sto, and P/Bi respectively.

Hybridizations were carried out and the hybridization products were analyzed by gel electrophoresis, according to the methods set forth in Example 1. Figure 5A shows the bands corresponding to each electrophoresed amplified sample. The gel bands were then Southern blotted onto membranes and probed with ^{32}P labelled probes 1-3. The membranes were washed twice for 30 minutes in 2X SSC buffer followed by two 30 minute washes in 0.2X SSC buffer. Wash temperatures were 67.3 degrees C (B31 group probe), 68.3 degrees C (P/Sto group probe), and 65.8 degrees C (P/Bi group probe). Further details of the Southern blot procedure are set forth in Picken, BioTechnique, 9:412 (1990). The results, shown in Figures 5B, C, and D, indicate that each probe is

completely specific for its corresponding subgroup of *B. burgdorferi* indicated in Table 1.

EXAMPLE 3

In a further series of experiments, DNA from a panel of *Borelia* was amplified using primers of complementary strand specificity flanking a sequence of *B. hermsii* in corresponding position to those described for *B. burgdorferi*. The first oligonucleotide primer was selected from the group consisting of sequences comprising about 8-32 substantially consecutive bases from nucleotide numbers 390 to 770 of the flagellin gene open reading frame. A second oligonucleotide primer was selected from the group consisting of sequences on the complementary strand located spacedly about 35 to 55 nucleotides from the 5' end of the first primer, comprising about 8-32 nucleotides having not greater than 70 percent homology with corresponding sequence of *B. burgdorferi*.

The primers utilized in these experiments are shown in Figure 9.

It was found that these primers amplified all the *B. hermsii* strains tested in addition to *B. parkeri* and *B. turicatae*. Upon sequencing the amplified DNA of the *B. parkeri* and *B. turicatae*, differences in the interprimer sequence were noted for these organisms compared to *B. hermsii*. These differences are shown in Figure 7. A probe was constructed selecting the sequence shown in Figure 7 in bold letters. After gel electrophoresis, the amplified bands were transferred to a membrane and Southern blotted

utilizing the probe. All procedures were as given in the preceding examples, except that the wash temperatures for the *B. hermsii* probe was 57.7 degrees C., and the wash temperature for the *B. parkeri/turicatae* probe (shown in Figure 7 as the bold letters of *B. hermsii* (HS1) with the bold substitutions at the nucleotide positions in which those strains (park and tri) differ from HS1) was 62.0 degrees C.

The probe sequence contains essentially a core sequence from nucleotide number 606 to 633, but bases may be added at either end, to stabilize hybrid formation, or to adjust the stringency of the wash procedures. The preferred probe thus comprises an oligonucleotide having the sequence TGC AGG TGA AGG CGC TCA GGC TGC T CCA GTG CAA GAG ATA conjugated to signal generating means such as ³²P.

Utilizing a probe having the nucleotide sequence of *B. hermsii* shown in bold letters in Figure 7, permitted the differential identification of *B. hermsii*. The Southern blot analysis shown in Figure 8 confirms the high specificity of the probe. This has clinical significance in that *B. hermsii*, which causes relapsing fever can be distinguished from the causative agent of Lyme Disease, and from *Borrelia* organisms causing animal disease.

INDEX TO LEGEND FOR FIGURE 5

Borrelia burgdorferi

- 1.* B31 [ATCC #35210]
2. IRS [ATCC #35211]
3. Virulent MM1

- 4. **Virulent MMT1**
- 5. **Virulent *I.pacificus***
- 6. **N40**
- 7. **Son 328**
- 5 8. **DN127**
- 9. **Minnesota Mouse**
- 10. **Lake 339**
- 11. **France 20001**
- 12. **CD16**
- 10 13. **VS215**
- 14. **NE56**
- 15. **Millbrook 25015**
- 16. **P/Sto**
- 17. **P/Gu**
- 15 18. **P/Bi**
- 19. **German Tick Isolate**
- 20. **123 Base Pair Ladder M. Wt. Marker**
- 21. **G2**
- 22. **VS3**
- 20 23. **VS185**
Borrelia hermsii
- 24. **HS1 [ATCC #35209]**
- 25. **Frogner**
- 26. **YOR-1**
- 25 27. **CON-1**
- 28. **MAN-1**
- 29. ***B. parkeri***
- 30. ***B. turicatae***
- 31. ***B. crocidurae***
- 30 32. ***B. anserina***
- 33. ***B. coriaceae***
- 34. ***Treponema pallidum***
- 35. ***Treponema pectinovorum***

36. *Treponema phagedenis*
37. *Leptospira interrogans* sv. *icterohemorrhagiae*
38. *Leptospira inadiei* sv. *lyme*
39. distilled water control
5 40. 123 Base Pair M. Wt. Marker
* Gel lane number

LEGEND TO FIGURE 8

Figure 8 shows the results of amplification from 13 different strains of *Borrelia* and 5 other strains of spirochetes using sequences derived from the flagellin gene of *B. hermsii*. The order of strains from left to right in each of the gel pictures 8A-8D is listed above/below.

- 10 8A. Agarose gel of the PCR products from 18 bacterial strains after amplification with the *B. hermsii* primer pair. Amplification is seen only for: *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. crocidurae*, *B. anserina* and *B. coriaceae*.
- 15 8B. A Southern blot of the gel shown in Figure 8A which has been probed with an oligonucleotide probe derived from the flagellin gene sequence of *B. hermsii*.
- 20 8C. A Southern blot of the gel shown in Figure 8A which has been probed with the oligonucleotide probe and in 8B but containing two redundancies.
- 25 8D. A Southern blot of the gel shown in Figure 8A which has been probed with an oligonucleotide probe derived from the flagellin gene sequence of *B. parkeri* and *B. turicatae*.

30 INDEX TO LEGEND FOR FIGURE 8

- 1.* *B. burgdorferi* B31
2. *B. burgdorferi* P7Sto
3. *B. Burgdorferi* P/Bi

4. *B. hermsii* HS1
 5. *B. hermsii* Frogner
 6. *B. hermsii* YOR-1
 7. *B. hermsii* CON-1
 - 5 8. *B. hermsii* MAN-1
 9. *B. parkeri*
 10. *B. turicatae*
 11. *B. crocidurae*
 12. *B. anserina*
 - 10 13. *B. coriaceae*
 14. *B. pallidum*
 15. *T. phagedenis*
 16. *T. denticola*
 17. *L. interrogans* sv. *icterohemorrhagiae*
 - 15 18. *L. inadiei* sv. *lyme*
 19. distilled water control
 20. 123 Base Pair M. Wt. Marker
- * Gel lane number

WHAT IS CLAIMED IS:

1. Primers of complementary strand specificity flanking a sequence of *B. burgdorferi* to be amplified by nucleic amplification techniques, said primers comprising

5 a first oligonucleotide selected from the group consisting of sequences comprising about 8-32 substantially consecutive bases from nucleotide numbers 390 to 770 of the flagellin gene open reading frame, and

10 a second oligonucleotide selected from the group consisting of sequences on the complementary strand located spacedly about 35 to 55 nucleotides from the 5' end of the first said primer, comprising about 8-32 substantially consecutive bases having not greater than 70 percent homology with the corresponding sequence of *B. hermsii*.

2. An oligonucleotide tracer for detection of *B. burgdorferi* target sequences comprising

5 an oligonucleotide probe sequence substantially homologous to a sequence of the flagellin gene open reading frame of *B. burgdorferi*, but not greater than 70 percent homologous to the corresponding sequence of *B. hermsii*, and

 signal means conjugated to said probe.

3. A bioassay for detection of *B. burgdorferi* in a biological sample comprising

5 extracting the nucleic acid fraction, denaturing the nucleic acids in the presence of primers of complementary strand specificity to sequences flanking a

- semiconserved region of nucleic acid encoding
the flagellin open reading frame,
hybridizing said primers to said
complementary strand sequences,
amplifying the interprimer sequence by
nucleic acid amplification techniques,
hybridizing the amplified interprimer
sequence to an oligonucleotide tracer
comprising a probe having a sequence
substantially homologous to a corresponding
sequence of the flagellin open reading frame
of *B. burgdorferi* but not greater than about
70 percent homologous to the corresponding
sequence of *B. hermsii*, and signal generating
means conjugated thereto, and
detecting a signal emitted by said
signal-generating means conjugated to said
hybridized tracer.
4. A substantially homogeneous assay for
detection of *B. burgdorferi* nucleic acid
sequences comprising
denaturing the nucleic acid sequences in
the presence of primers of complementary
strand specificity to sequences flanking a
semiconserved region of nucleic acid encoding
the flagellar open reading frame,
hybridizing said primers to said
complementary strand sequences,
amplifying the interprimer sequence by
nucleic acid amplification techniques,
hybridizing the amplified interprimer
sequence to an oligonucleotide tracer
comprising a probe having a sequence

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substantially homologous to a corresponding sequence of the flagellin open reading frame of *B. burgdorferi* but not greater than about 70 percent homologous to the corresponding sequence of *B. hermsii*, and a fluorescent tag conjugated thereto, and

measuring the increase in fluorescence polarization.

5

5. A variant sequence of the *B. burgdorferi* flagellin gene open reading frame hypervariable region extending from nucleotide number 517 to number 742 comprising base substitutions of a guanosine at positions 531, 549, 552, 612, 613, 684, 693, adenosine at positions 539, 591, 603, 622, 639, 651, 666, thymidine at positions 540, 573, 630, 696, and cytosine at position 735.
6. A variant sequence of the *B. burgdorferi* flagellin gene open reading frame hypervariable region extending from nucleotide number 517 to number 742 comprising base substitutions of a guanosine at positions 531, 595, 612, 649, and 663, adenosine at positions 539, 552, 651, 666, 670, and 688, thymidine at positions 540, 571, 594, 630, and 696, cytosine at positions 644 and 726.
7. An oligonucleotide tracer for detection of subgroups of *B. burgdorferi* comprising an oligonucleotide probe having a sequence selected from the group consisting of CTC TGG TGA GGG AGC TCA AAC TGC TCA GGC TGC ACC GGT TCA AGA GGG T, CTC TGG TGA AGG AGC TCA GGC TGC TCA GAC TGC ACC TGT TCA AGA AGG, and

5

5

TGC TGG TGA GGG AGC TCA AGC TGC TCA GGC TGC
ACC TGT TCA AGA GGG TGC, and

10 signal means conjugated to said probe.

8. Primers of complementary strand specificity flanking a sequence of *B. hermsii* to be amplified by nucleic acid amplification techniques, said primers comprising

5 a first oligonucleotide selected from the group consisting of sequences comprising about 8-32 substantially consecutive bases from nucleotide numbers 390 to 770 of the flagellin gene open reading frame, and

10 a second oligonucleotide selected from the group consisting of sequences on the complementary strand located spacedly about 35 to 55 nucleotides from the 5' end of the first said primer, comprising about 8-32 substantially consecutive bases having not greater than 70 percent homology with the corresponding sequence of *B. burgdorferi*.

15

9. An oligonucleotide tracer for specific detection of *B. hermsii* comprising
 - an oligonucleotide probe having a core sequence extending from nucleotide 606 to 633 of the *B. hermsii* flagellin gene open reading frame, and
 - signal-generating means.

FIG. 1A

1 ATGATTATCAAATCATAATACATCAGCTTAAATGCTTCAGAAATAATGG 50
1 ATGATCATAATCATAATACGTCACTAGCTTAAATGCTTCAGAAATAATAG 50

51 CATTAAACGCCGCTTAATCCTTAGTAAAACCTCAAGAAAAGCTTCTAGTGGGT 100
51 CATTAAATGCTACTTAATCCTTAGCAAAACTCAAGAAAACCTTCTAGTGGGC 100

101 ACAGAAATTAAATCGAGCTTCTGATGCTGCTGGCATGGGAGTTCTGGT 150
101 ATAGAAATTAAATCGTGCATCTGATGCTGCTGGTATGGCCGTTGGTGA 150

151 AAGATTAATGCTCAAAATAAGGGTTTGTCAAAAGCTTCTAGAAATACTTC 200
151 AAAATTAAATGCTCAAATTAGGGTTCTAGGCTTCTAGAAATACTTC 200

201 AAAGGCTTAAATTATTCAAGACAACAGAAAGGAATTAAATGAAGTAG 250
201 AAAGGCTTAAATTATTCAACAAACAGAAAGGAATTAAATGAAGTAG 250

251 AAAAAGTCTTAGTAAGGAATTGGCAGTTCAATCAGGTAAACGGC 300
251 AGAGAGTATTAGTAAGGAATGAAAGAACTTGCTGTTCAATCTGGTAATGGT 300

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301 ACATATTCAAGATGCCAGACAGGGTTCTATAAAATTGAAATAGAGCAACT 350
 301 ACATATTCAAGATGCCAGACAGGGTTCTATAAAATTGAAATGAGCAACT 350
 351 TACAGACCAAATTAAATAGAATTGCTGATCCAAGCTCAATAAACCAAATGC 400
 351 TACAGATGAAATTCAACAGAAATTGCTGATCCAAGCTCAATAAACCAAATGC 400
 401 ACATGTTATTCAAAACAAATCTGCTTCTCAAAAATGTAAGAACAGGCTGAAGAG 450
 401 ATATGTTGTCCAAACAAAGTCAGCTGCTCAAAATGTAAAAACAGGCTGAAGAG 450
 451 CTTGGAATGCCAGCCTGCAAAATTAAACACACCAGCATCACTTTCAGGGTC 500
 451 CTTGGAATGCCAACCGCAAAATTAAACACACCAGCATCACTAGCTGGATC 500
 501 TCAAGGGTCTTGACTTTAAGAGTTCATGTTGGAGCAACCCAAGATGAAG 550
 501 ACAAGCTTCATGGACATTGAGAGTACATGTTGGGGAAATCAGGATGAGG 550
 551 CTATTGCTGTAATTATTATGCCAGCTTAATGTTGCAAATCTTTCTCTGGT 600
 551 CAATTGCTGTTAATTATTATGCCATCTTAATGTTGCAAATCTTTTGAGGT 600
 601 GAGGGAGGCTCAAACCTGCTCAGGGTGGCACCCGGTCAAGAGGGTCTCAACA 650
 601 GAAGG.....CGCTCAGGTGCTCCAGTGCIAAGAGATAGGACAGCA 641

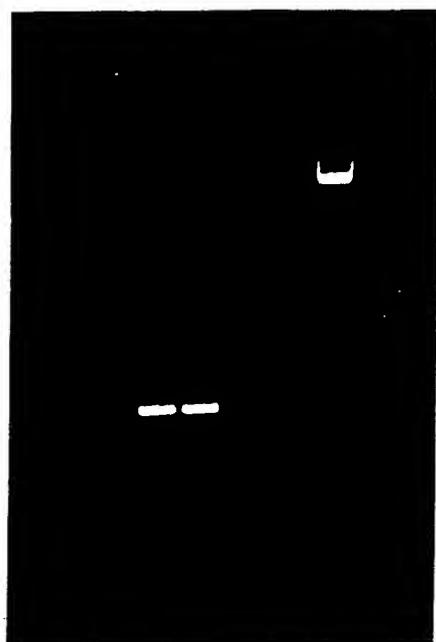
FIG. 1B

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651	GAAGGAGCTAA. CAG.. CCAGCACCTGCTACAGCACCTTCTCAAGGCC	697
642	AGAGGAAGGTCAAGCAGCTCCAGCTCCAGGAGGCTCAAGGTG	691
698	GAGTTAARTCTCTGTTAACACTACAGTTGATGCTAACAT <u>ATCA</u>	747
692	GAGTTAATTCCCATAATTAAATGTTACAACCCTGTTGATGCTAACATGTCA	741
748	<u>CTTGCTAAATTGAAATGCTTATTAGAATGATAAGTGTCAAAAGGCCAA</u>	797
742	CTTGCAAAGATAGGGTGTGCTATTAGGATGTAAGTGTCAAAAGGCCAA	791
798	TTTAGGTGCTTTCCAATAATAGACTTGAATCTATAAAGAAATAGTACTGAGT	847
792	TCTTGGTGTTCAAAAGCATCTTATGCTCAAAATAAAAGATGCTACA	841
848	ATGCAATTGAAATCTAAAGCATCTTATGCTCAAAATAAAAGATGCTACA	897
842	ATGCTATTGAAACTTGAAGGCATCATATGCTCAAATTAAAGATGCAACCA	891
898	ATGACAGATGAGGTGTGAGGCCAACTAATTAGTATTAAACAAATC	947
892	ATGACAGATGAAAGTGTGAGCATCAACACTCACAGTATTGACACAAATC	941
948	TGCAATTGCAATTGCTGGCAGGGTAATCAAGTTCCCAATATGTTTGT	997
942	TGCAATTGCTATTGCTGCAACAAATCAAGTACCTCAAATATGTTTAT	991
998	CATTGCTTAGATAA	1011
992	CATTGCTTAGATAA	1005

FIG. I C

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AGAROSE GEL

1 2 3 4 5 6

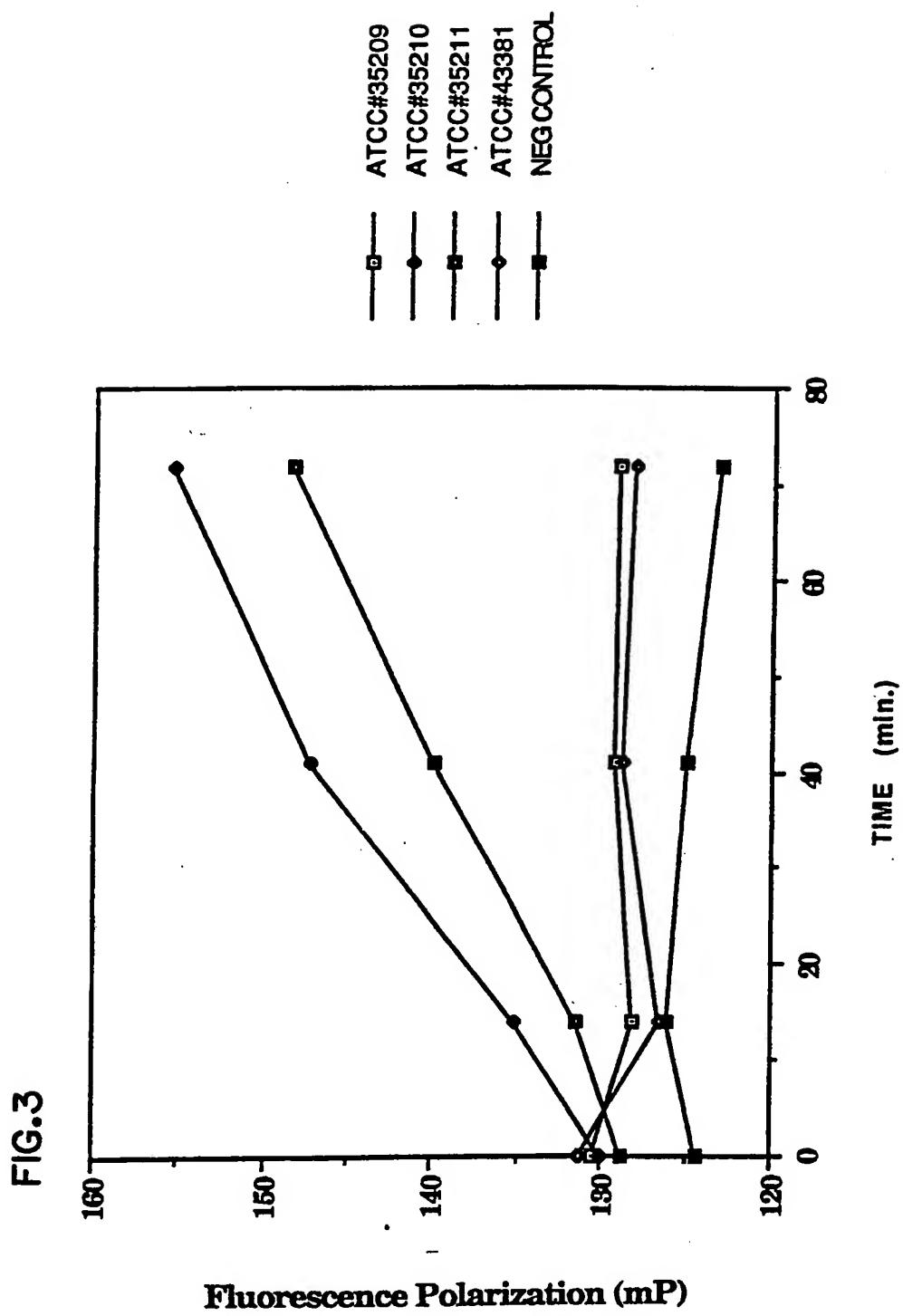


SOUTHERN BLOT

FIG.2

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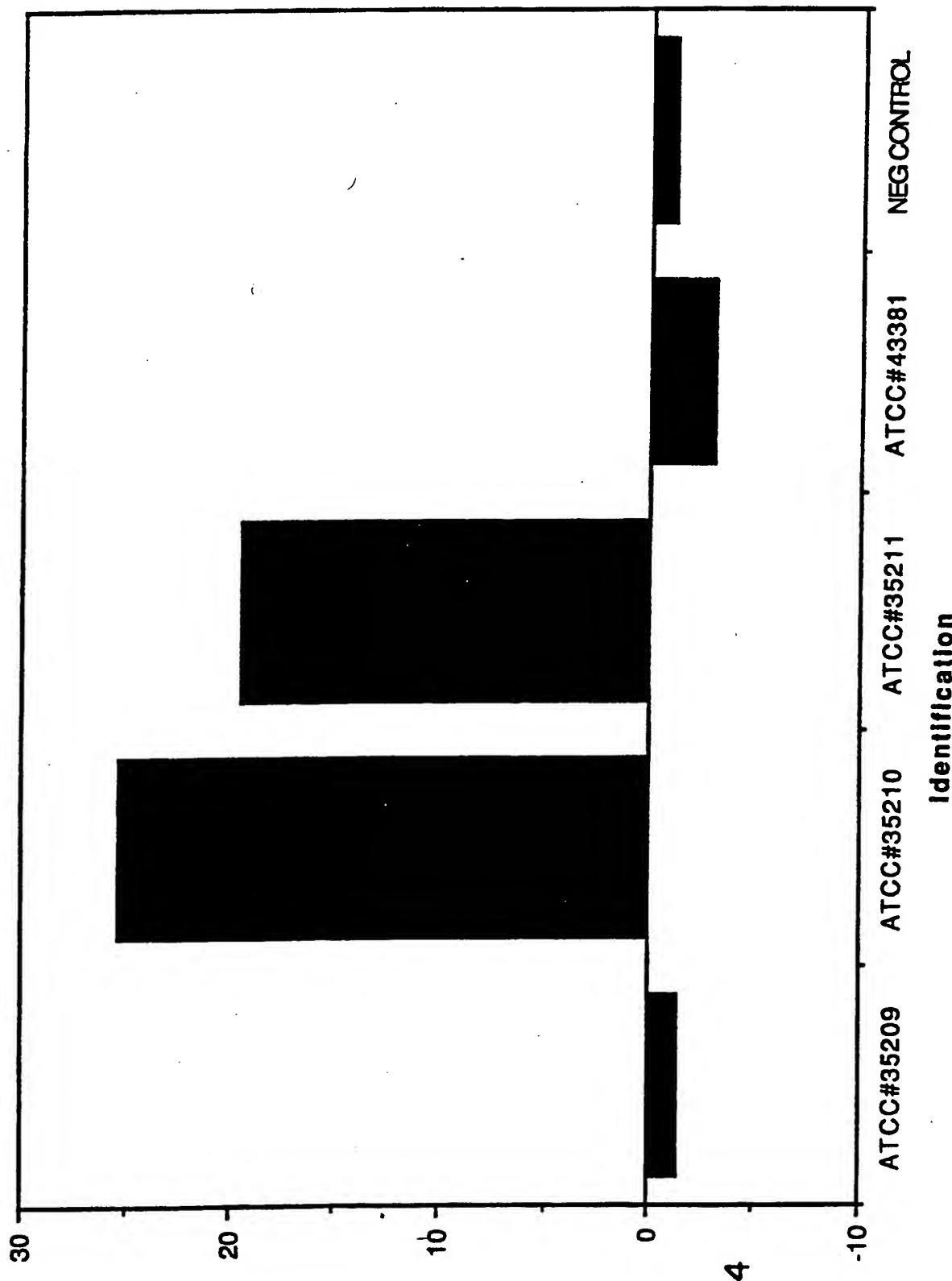


FIG. 4

Delta mP (at 72 min.)

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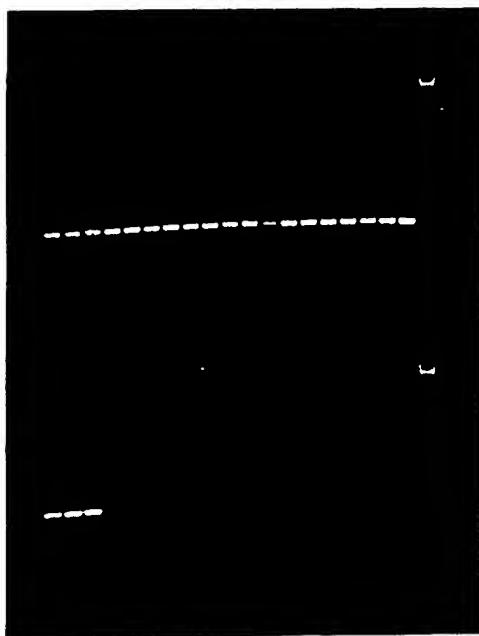


FIG.5A

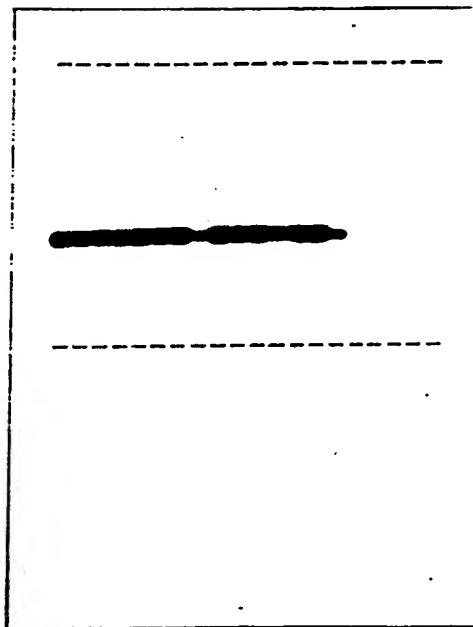


FIG.5B

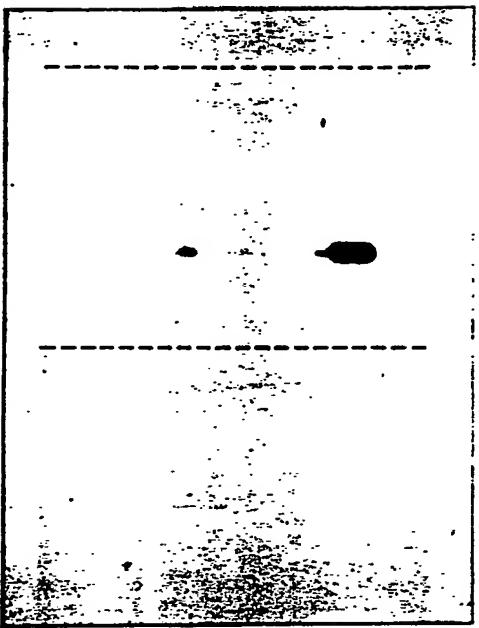


FIG.5C

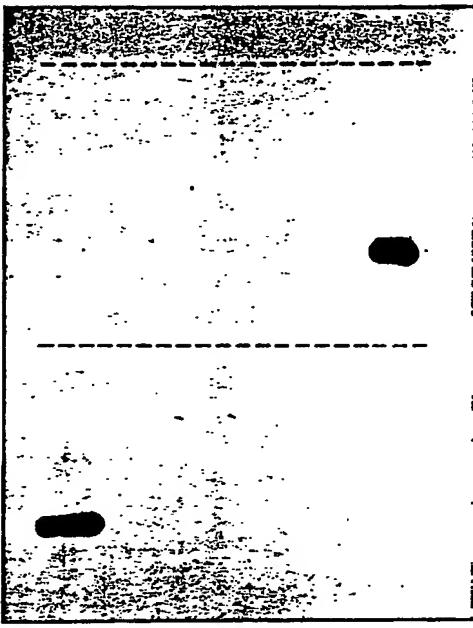


FIG.5D

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FIG.6A

B31 25015	517	TTAAGGAGTC ATGTTGGAGC AACCCAAAGAT GAAGCTATG CTGTAAATAT	566
P/Bi		G-----AT-----G-----G-----G-----	
G1		G-----AT-----G-----G-----G-----	
G2		G-----AT-----G-----G-----G-----	
VS3		G-----AT-----G-----G-----G-----	
VS185		G-----AT-----G-----G-----G-----	
P/Sto		G-----AT-----A-----AT-----A-----	
P/Gu		G-----AT-----A-----AT-----A-----	
B31 25015	567	TTATGCAGCT AATGTTGCCAA ATCTTTCCTC TGCTGAGGGAA GCTCAAAACTG	616
P/Bi		T-----A-----A-----A-----GG-----	
G1		T-----A-----A-----A-----GG-----	
G2		T-----A-----A-----A-----GG-----	
VS3		T-----A-----A-----A-----GG-----	
VS185		T-----A-----A-----A-----GG-----	
P/Sto		T-----TG-----TG-----G-----	
P/Gu		T-----TG-----TG-----G-----	

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B31	617	CTCAGGCTGC	ACCGGTTCAA	GAGGGTTC	ACAGGAAGG	AGCTCAACAG	666
25015		-----T-----	-----A-----	-----G-----A-----	-----A-----	-----A-----	
P/B1		-----A-----	-----T-----	-----A-----	-----A-----	-----A-----	
G1		-----A-----	-----T-----	-----A-----	-----A-----	-----A-----	
G2		-----A-----	-----T-----	-----A-----	-----A-----	-----A-----	
V3		-----A-----	-----T-----	-----A-----	-----A-----	-----A-----	
VS185		-----A-----	-----T-----	-----A-----	-----A-----	-----A-----	
P/Sto		-----T-----	-----C-----	-----G-----A-----	-----G-----A-----	-----G-----A-----	
P/Gu		-----T-----	-----C-----	-----G-----A-----	-----G-----A-----	-----G-----A-----	
B31	667	CCAGCACCTG	CTACAGCACC	TTCCTCAAGGC	GGAGTTAATT	CTCCTGTAA	716
25015		-----A-----	-----T-----	-----G-----T-----	-----G-----T-----	-----G-----T-----	
P/B1		-----A-----	-----G-----	-----G-----T-----	-----G-----T-----	-----G-----T-----	
G1		-----A-----	-----G-----	-----G-----T-----	-----G-----T-----	-----G-----T-----	
G2		-----A-----	-----G-----	-----G-----T-----	-----G-----T-----	-----G-----T-----	
V3		-----A-----	-----G-----	-----G-----T-----	-----G-----T-----	-----G-----T-----	
VS185		-----A-----	-----G-----	-----G-----T-----	-----G-----T-----	-----G-----T-----	
P/Sto		-----A-----	-----A-----	-----A-----T-----	-----A-----T-----	-----A-----T-----	
P/Gu		-----A-----	-----A-----	-----A-----T-----	-----A-----T-----	-----A-----T-----	
B31	717	TGTACACT	ACAGTTGATG	CTAATA	742		
25015		-----C-----	-----C-----	-----C-----	-----C-----	-----C-----	
P/B1		-----C-----	-----C-----	-----C-----	-----C-----	-----C-----	
G1		-----C-----	-----C-----	-----C-----	-----C-----	-----C-----	
G2		-----C-----	-----C-----	-----C-----	-----C-----	-----C-----	
V3		-----C-----	-----C-----	-----C-----	-----C-----	-----C-----	
VS185		-----C-----	-----C-----	-----C-----	-----C-----	-----C-----	
P/Sto		-----C-----	-----C-----	-----C-----	-----C-----	-----C-----	
P/Gu		-----C-----	-----C-----	-----C-----	-----C-----	-----C-----	

FIG.6B

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HS1 517 TTGAGACTC ATGTGGCGC AAATCAGGAT GAGGCAATTG CTGTTAATA 566
 YOR-1 -----A----- -----A----- -----A----- -----A----- -----A-----

park
turi
-----A----- -----T----- -----A----- -----A----- -----A-----

HS1 567 TTATGCATCT AATGTGCAA ATCTTTTTCG AGGTGAAGGC GCTCAGGCTG 616
 YOR-1 -----A----- -----A----- -----C----- -----T----- -----T-----

park
turi
-----G----- -----C----- -----C----- -----T----- -----G----- -----T-----

HS1 617 CTCAGTGCA AGAGATAGGA CAGCAAGAGG AAGGTCAAGC AGCTCCAGCT 666
 YOR-1 -----A----- -----A----- -----A----- -----A----- -----A-----

park
turi
-----CT-----G--AGGT-C-----A-----G--T-----T-----A
-----CT-----G--AGGT-C-----A-----G--T-----T-----A
-----A-----G-----T-----A-----G-----T-----A

HS1 667 CCAGCAGC CTCCAGCTCA AGGTGGAGTT ATTCCCCAA TTAATGTAC 716
 YOR-1 -----G----- -----G----- -----T----- -----T----- -----T-----

park
turi
-----A----- -----A----- -----A----- -----T----- -----G----- -----G-----

HS1 717 AACCGCTGTT GATGCTAATA 736
 YOR-1 -----A----- -----A----- -----A----- -----A----- -----A-----

park
turi
-----TA----- -----TA----- -----A----- -----A----- -----A-----

FIG. 7

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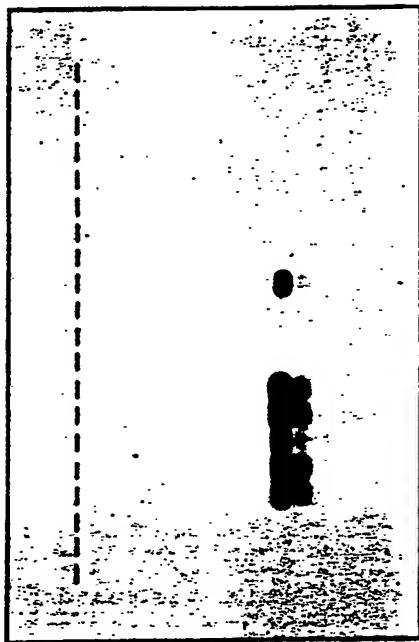


FIG. 8B

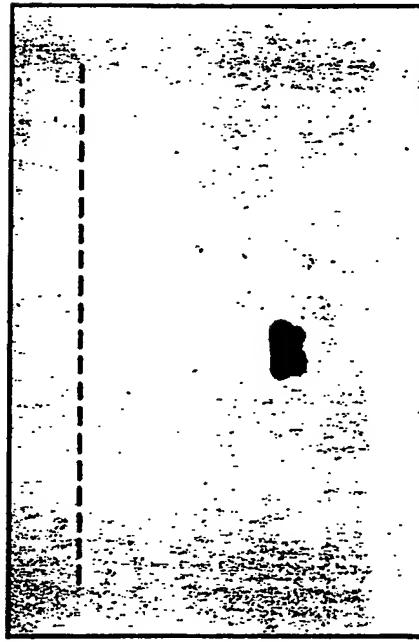


FIG. 8D

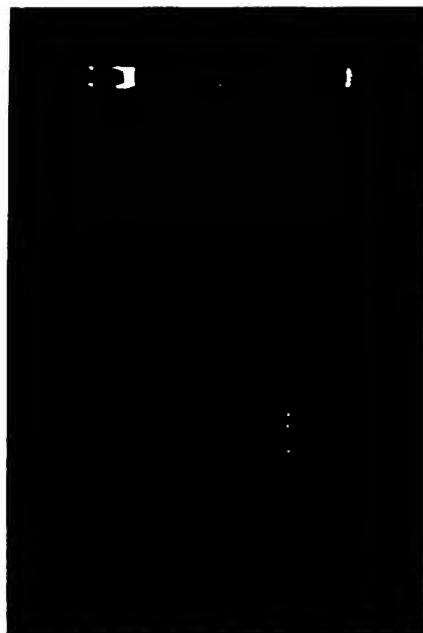


FIG. 8A

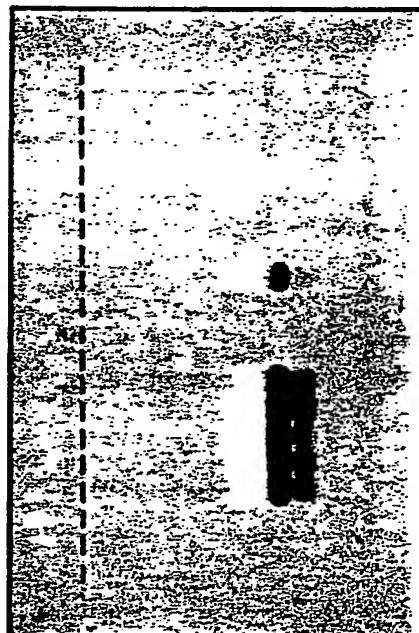
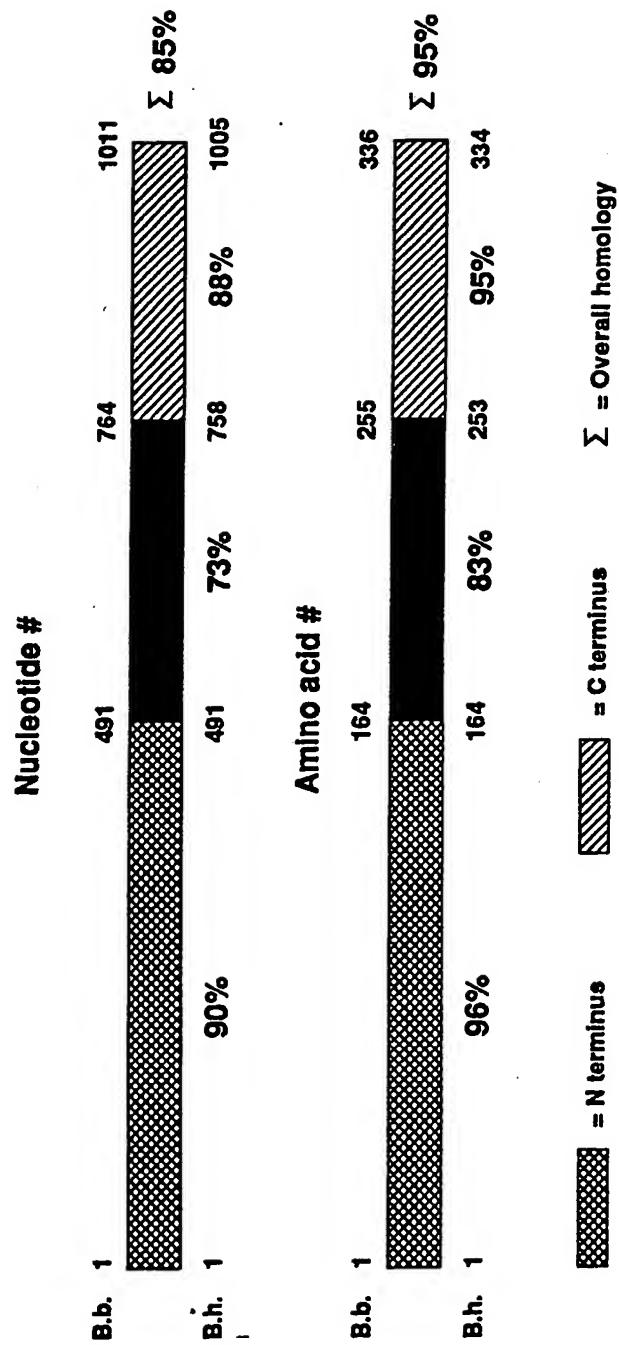


FIG. 8C

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FIG.9A



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492 TTCAGGGTCAAGCGTCTGGACT TAAGAGTTCATGTTGGAGCAACCC 541
 492 AGCTGGATCACAAAGCTCATGGACAT GTGAGAGTACATGTGGGCAAATC 541
 542 AAGATGAAGCTATTGCTGTAATAATTATGCAAGCTTAATGTTGCAAATCTT 591
 542 AGGATGAGGCCAATTGCTGTTAATAATTATGCATCTAATGTTGCAAATCTT 591
 592 TTCCTGGTGAGGGAGCTCAAACTGCTCAGGGCTGCA
GGCTCAAGAGGG 641
 592 TTGGCAGGTGAAGGG GCTCAGGGCTGCTCCAGTGCAAGAGAT 632
 642 GTTCAACAGGAAGGGCTCAA. CAG.. CCAGGCACCTGCTACAGCACCTT 688
 633 AGGACAGCAAGAGGAAGGTCAAGCAGCTCCAGCTCCAGCAGCTCCAG 682
 689 CTCAGGGGAGTTAATTCTCTGTTAATGTTACAACACTACAGTTGATGCT 738
 683 CTCAAGGGGAGTTAATTCCCCAATTAAATGTTACAAACCCTGTTGATGCT 732
 739 AATAACATCACTTGCTAAATTGAAATGC 767
 733 AATAATGTCACTTGCAAAGATAAGGG GC 761

FIG.9B